

Rediscovery of *Roesleria subterranea* from Japan with a discussion of its infraspecific relationships detected using molecular analysis

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Abstract

Roesleria subterranea, a distinctive hypogeous fungus, was collected from unidentified deciduous plant roots in red pine forests. The fungus had been documented several times in the past in Japan, but with no description. A description is given here based on specimens collected in Japan. The sequence of the D1-D2 region of the 28S rDNA obtained from the isolate was identical to those of the European and American specimens. Maximum parsimony analysis incorporating the present data and all other available ITS-5.8S sequences for *R. subterranea* showed that there are two infraspecific groups. One of them, composed of the isolates from *Vitis* spp. in Germany, Italy, and USA, was monophyletic. The other group, composed of isolates from deciduous trees in various countries, including Japan, was paraphyletic. The phylogenetic patterns indicate that the host may be more important than geographical distance for the genetic diversification of *R. subterranea*.

Key words

Geographic distribution, Helotiales, Hypogeous fungi, ITS-5.8S, Taxonomy

Introduction

Roesleria subterranea (Weinm.) Redhead is a distinctive hypogeous fungus currently placed in Roesleriaceae (Yao and Spooner 1999) or Helotiaceae Helotiales (Neuhauser et al. 2011). It is recognized as a facultative root parasite that causes damage to grape vines (Neuhauser et al. 2011). Ecologically, it occurs on the plant roots of various deciduous trees. Its morphology characterized by stalked apothecia with spores produced in evanescent asci in dried masses (mazaedia), shows a similarity to mazaediate lichens such as *Calicium* Pers. It is closely related to helotiaceous fungi, in particular *Hymenoscyphus* Gray and *Cudoniella* Sacc. (Kirchmair et al. 2008).

Roesleria subterranea is distributed primarily in Europe and North America, with rare reports from Asia. In Japan, three presumptive occurrences of *R. subterranea* have been reported. *Roesleria hypogaea* Thüm. & Pass. was first documented in Japan by Shirai (1894) as a possible agent of root rot in grapes. Hara (1930) later cited the fungus as of an uncertain occurrence in Japan. Togashi (1950) listed the name *Coniocybe pallida* (Pers.) Fr. as a causative agent of grape root rot and reported its occurrence in Hokkaido and Akita prefecture. This name was cited in fungi from the Tohoku area (North East of Japan) by Sawada (1952) as a synonym of *Roesleria pallida* (Pers.) Sacc., (Sawada 1952, p. 143). The fungus was thought to be a root rot agent of *Vitis vinifera* in Morioka. Currently, *Roesleria hypogaea* is treated as a synonym of *R. subterranea*, while *R. pallida* is recognized as *Sclerophora pallida* (Fr.) Yao & Spooner (Coniocybaceae based on *Calicium pallidum* Fr.), which was revealed to be a lichen name (Redhead 1984, Yao and Spooner 1999). Therefore, *R. subterranea* occurs in the northern part of Japan, but none of the authors provided a definite description of the species, nor are voucher specimens available.

A specimen of *R. subterranea* was recently collected from unknown deciduous tree roots in the red pine forests in Sugadaira heights, Nagano prefecture, Japan, from which an isolate was obtained. Because *R. subterranea* is a subterranean fungus with a unique phylogenetic position as a helotiaceous fungus, it is documented here with molecular data and reference to the specimens in Japan.

Materials and methods

Collection and isolation

A specimen was collected on roots of unknown trees beneath the log of *Pinus densiflora*, among the conifer forests of 30-year-old trees mixed with other deciduous trees such as *Betula platyphylla*, *Cerastrus orbiculatus*, *Ligustrum tschonoskii*, *Rhododendron japonicum*, *Swida controversa*. Although *Vitis coignetiae* is present in this forest, no individual was found nearby the collection site of the fungus, so it seems likely plausible that the host was not *Vitis*. The material was air-dried at 23°C for 24 hours. A small amount of spores were picked up by a fine needle and transferred to plates of malt extract agar (MEA; Nissui, Tokyo, Japan), cornmeal agar (CMA; Nissui), and half-strength malt extract-yeast extract agar (MEYE; malt extract 3 g; yeast extract 3 g, peptone 5 g, dextrose 10 g,

agar 15 g, DW 1 L). Germination was observed under a light microscope to obtain a pure culture. The specimen and isolate were deposited in the mycological herbarium of the National Museum of Nature and Science (TNS) and the National Institute of Technology and Evaluation, Biological Resource Center (NBRC). The color names and codes in the description followed the Pantone color bridge (Pantone Inc., Carlstadt, NJ, USA), adopting the CYMK color system. Additional specimens collected in Japan were investigated in the TNS fungal herbarium.

DNA extraction, polymerase chain reaction (PCR), and sequencing

The isolates were incubated in 2% malt extract broth for 2 weeks, and the mycelium was harvested. Approximately 50 mg of mycelium was mechanically lysed by a Qiagen Tissue Lyser Kit (Qiagen Inc., Mississauga, ON, Canada), using ceramic beads following the manufacturer's instructions. The DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc.) following the manufacturer's instructions. To amplify the internal transcribed spacer (ITS1 and ITS2) and 5.8S rDNA regions (ITS-5.8S), the primer pair ITS1F and ITS4 (White et al. 1990) was used. To amplify the D1-D2 region of 28S rDNA (nLSU rDNA; D1-D2), the primer pair NL1 and NL4 (O'Donnell 1993) was used. The DNA was amplified using 40- μ l PCR reactions containing 0.2 μ M of each primer, 1 unit of TaKaRa Ex Taq DNA polymerase (Takara Bio, Otsu, Japan), and a deoxynucleoside triphosphate (dNTP) mixture containing 2.5 mM of each dNTP and ExTaq buffer containing 2 mM MgCl₂. PCR was carried out using a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The DNA was denatured for 3 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. Residual primers, and unincorporated dNTPs were removed to prevent the inhibition of the following sequencing reaction using a ExoSAP-IT purification kit (USB Corp., Cleveland, OH, USA). Total DNA samples extracted for the present study were deposited in the Center for Molecular Biodiversity Research in National Museum of Nature and Science (ED16207). The sequencing reaction was carried out using a Big-Dye terminator cycle sequencing kit (Applied Biosystems) following the manufacturer's instruction using the same primers as those used for DNA amplification. Sequencing was conducted using an ABI 3130x Genetic Analyzer (Applied Biosystems).

Molecular phylogenetic analysis

The available sequences for *Roesleria subterranea* were obtained from GenBank (Table 1). The obtained sequence data for the ITS-5.8S region were aligned by Clustal W (Thompson et al. 1994) and edited manually when necessary using BioEdit v.7.0.5.2 (Hall 1999). Phylogenetic analysis was conducted by a maximum parsimony (MP) method. An MP heuristic search was carried out using the PAUP* version 4b10 (Swofford 2002) with 1,000 replications, each with the option of random sequence additions. Branch

Table 1. Sequences used in the present study. New sequences generated for this analysis are in bold.

Name	ID of the isolate	ITS-5.8S	28S rDNA*	Origin	Country
<i>Roesleria subterranea</i>	NBRC108276	AB628057	AB628056	on roots of deciduous plant	Japan
<i>Roesleria subterranea</i>	CBS 271.82	EF060309		on roots of <i>Populus</i> sp.	Netherlands
<i>Roesleria subterranea</i>	CBS 339.96	EF060308	EF608074	buried twig of deciduous shrub	Austria
<i>Roesleria subterranea</i>	CBS 320.33	EF060307		on roots of <i>Malus sylvestris</i>	Netherlands
<i>Roesleria subterranea</i>	CBS 201.25	EF060306		on roots of <i>Vitis vinifera</i>	USA
<i>Roesleria subterranea</i>	IB 2005/504	EF060305		“ <i>V. berlandieri</i> × <i>V. viparia</i> ”	Germany
<i>Roesleria subterranea</i>	IB 2005/511	EF060304		on roots of <i>V. berlandieri</i> × <i>V. viparia</i>	Germany
<i>Roesleria subterranea</i>	IB 2005/510	EF060303		on roots of <i>V. berlandieri</i> × <i>V. viparia</i>	Germany
<i>Roesleria subterranea</i>	IB 2005/507	EF060302		“ <i>V. berlandieri</i> × <i>V. viparia</i> ”	Germany
<i>Roesleria subterranea</i>	IB 2005/505	EF060301		on roots of <i>V. berlandieri</i> × <i>V. viparia</i>	Germany
<i>Roesleria subterranea</i>	IB 2005/506	EF060300	EF608075		Germany
<i>Roesleria subterranea</i>	IB 2005/508	EF060299		on roos of <i>V. rupestris</i> × <i>V. riparia</i>	Germany
<i>Roesleria subterranea</i>	CBS 407.51	EF060298	EF608073		Italy
<i>Hymenoscyphus epiphyllus</i>	isolate 1489	AY348580			
<i>Hymenoscyphus immutabilis</i>	isolate 71809	AY348584			
<i>Hymenoscyphus scutula</i>	MBH29259	AY789432			

swapping by tree bisection-reconnection (TBR) and MulTrees were in effect. Support for the individual nodes was tested with bootstrap analysis under the equally weighted parsimony criterion. The bootstrap analysis was based on 1000 bootstrap replicates using the heuristic search option (TBR and MulTrees options, on) of ten replicates with random addition sequences. Based on the previous analysis (Kirchmair et al. 2008), *Hymenoscyphus epiphyllus* (Pers.) Rehm ex Kauffman (AY348580), *H. immutabilis* (Fuckel) Dennis (AY348584), and *H. scutula* (Pers.) W. Phillips (AY789432) were used as outgroups. The trees were visualized using the Treeview program, version 1.6.6 (Page 1996). The alignments were deposited into TreeBASE as TB2:S11877.

Results and discussion

Description and taxonomy

Roesleria subterranea (Weinm.) Redhead, Can. J. Bot. 62: 2516, 1984.

Figs 1, 2

≡ *Pilacre subterranea* Weinm., Flora 15 (Beiblatt, Bd. 1), p. 458, 1832.

Note. For the detailed synonymy, see Redhead (1984).

Description. Apothecia capitate, long-stalked, 0.5–1.5 mm high, hypogeous, on plant root; head spherical to irregular, 0.4 mm diam., composed of dried spore mass (mazaedia), powdery, grayish green (C16M0Y36K0); stalk 0.1 mm diam., base of apothecium with elongated cells ($7\text{--}13 \times 2.5\text{--}4.5 \mu\text{m}$), pale-colored; ectal excipulum not observed due to maturation of apothecia. Asci $40\text{--}55 \times 6.5\text{--}7.0 \mu\text{m}$, cylindrical-clavate, thin walled, arising from croziers, eight-spored, evanescent, releasing ascospores by degradation of ascus wall; apex rounded, Meltzer reaction— with or without KOH pretreatment. Ascospores $5\text{--}6.4 \times 4.5\text{--}5.5 \mu\text{m}$, broadly elliptical, almost hyaline, pale-colored to dark-colored due to maturation, aseptate when young, becoming almost spherical to lenticular, transversely one-septate. Germination tubes usually occur from each cell of ascospores. Paraphyses cylindrical, simple, straight to waving, enlarged toward apex up to $6\text{-}\mu\text{m}$ wide, multiseptate, often long, extending beyond asci. Ascospores germinated on CMA, MEA, and MEYE to produce mycelium. On PDA, colonies of 65 mm diam in 30 days at 23 C, surface floccose; aerial mycelium well-developed at center, white; substratal mycelium dark green (C24M5Y98K35), becoming paler (C10M0Y54K0) toward the margin. Colonies were dark green (C24M0Y98K8) from the reverse, producing stronger colored patches, paler (C6M0Y54K0) toward margin. On MEA, colonies 65 mm diam in 30 days at 23 C, surface floccose; aerial mycelium well developed, light green (C10M0Y72K0) at center, becoming sparse and paler (C6M0Y54K0) toward margin; substratal mycelium obscured. Colonies dark green (C20M4K100Y32) at center from reverse, becoming paler (C6M0Y54K0) toward margin. On CMA, colonies 60 mm diam in 30 days at 23 C, mycelium sparse, with almost no coloration. Asexual state not observed in culture.

Specimens examined. Japan. Nagano Prefecture: Pinus densiflora forests approximately 30 years old, Sugadaira Montane Research Center, University of Tsukuba, Ueda ($36^{\circ}52.12'\text{N}$; $138^{\circ}34.97'\text{E}$ (DDM)), ca 1300–1360 m, 19 Nov 2010, Y. Degawa (TNS-F-38701), on unknown deciduous plant roots (not *Vitis*), multi-ascospore isolate FC-2678 (NBRC108276). Hokkaido isl., Sapporo: 20 Aug 1922, K. Togashi (TNS-F-185301) as *Calicium pallidum*, on *Vitis vinifera*. Iwate Prefecture: Morioka, Oct 1935, “College Orchard”, “Murata” (TNS-F-185302) as *Calicium pallidum*, on *Vitis vinifera* (The words with double quotation indicate the data written on the original specimen label. The precise information is unclear, as this specimen was donated to TNS by K. Togashi.).

The specimen collected at Sugadaira showed similar morphological agreement with previous reports (Redhead 1984, Yao and Spooner 1999, Kirchmair et al. 2008). The taxonomy of *R. subterranea*, however, has been under debate. It was suggested to belong to Caliciaceae, Caliciales (Redhead 1984). Yao and Spooner (1999) proposed Roesleriaceae, but its placement was not mentioned by Kirk et al. (2008). Based on the molecular phylogeny on rDNA, it was classified as Helotiaceae, but Roesleriaceae as a distinct family has not been ruled out (Kirchmair et al. 2008, Neuhauser et al. 2011).

Two other genera are known for their occurrence on plant roots. One is *Moserella* Pöder & Scheuer, known as a root endophyte of *Picea*, and the other is *Roeslerina* Redhead, obtained from coniferous roots (Redhead 1984, Yao and Spooner 1999). Because these two genera and *Roesleria* have mazaedia, mazaedia formation may be suggested to be a common convergent character in hypogeous apothecial fungi.

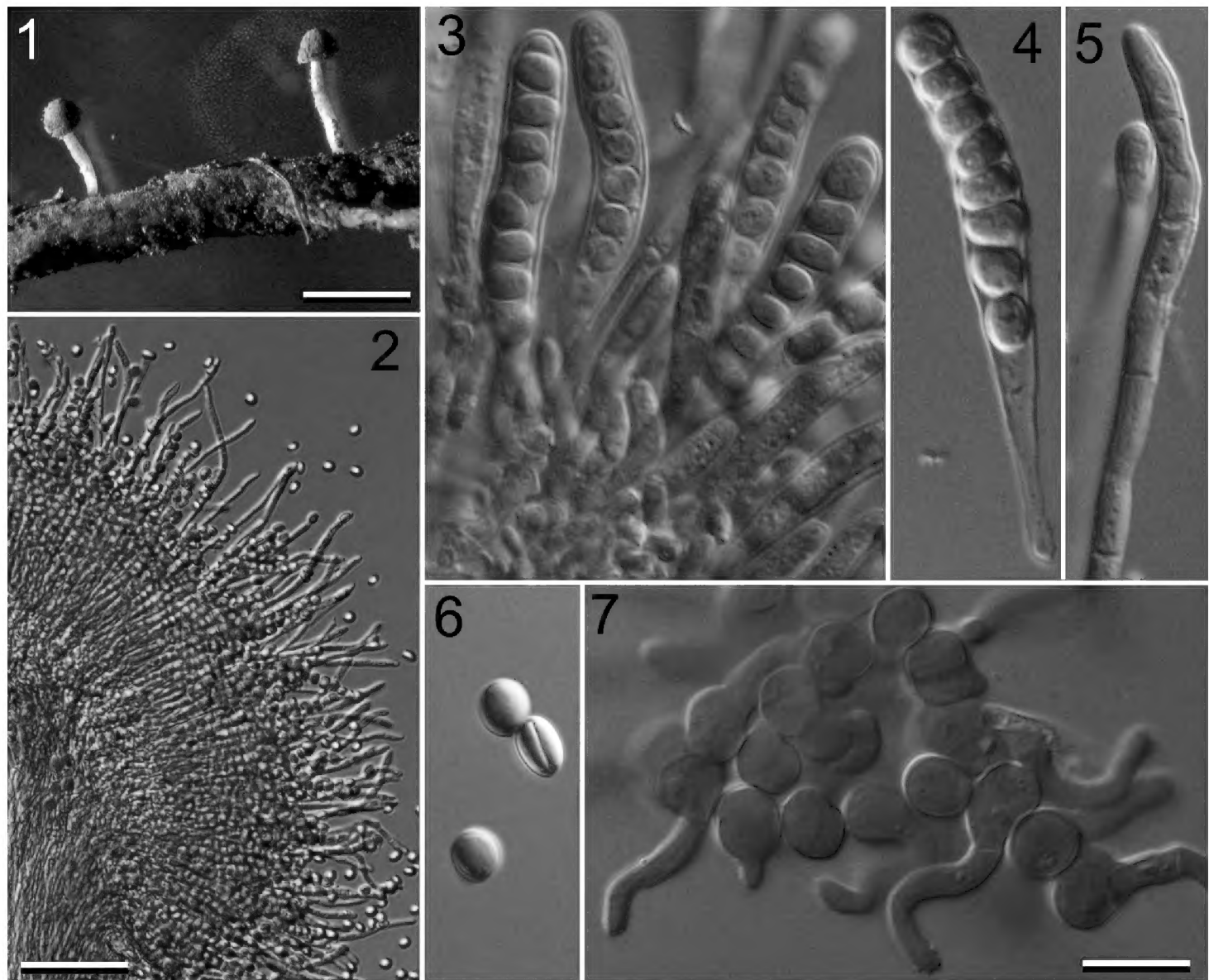


Figure 1. *Roesleria subterranea* (TNS-F-38701). **1** Growing habits of two mature mazaedia on the root tip of an unidentified tree **2** Hymenium of mazaedium, showing protruding paraphyses **3** Enlarged hymenium showing developmental stages of asci and paraphyses **4** Mature ascus containing eight ascospores **5** Paraphysis **6** Discharged matured ascospores; on the right, a transversely one-septate spore is shown in side view **7** Germination of ascospores on MEYE, showing that germination tubes from one of the two-celled spores **2–6** Mounted in Meltzer's solution **7** Mounted in lacto-aniline blue. Bars: **1** = 2 mm; **2** = 50 μ m; **3–7** = 10 μ m.

We found two specimens (TNS-F-185301 and 185302) of *R. subterranea* deposited in TNS as *Calicium pallidum*, which were reported by Togashi (1950). Although *Calicium pallidum* is a lichen name, it is also a misapplied name for *R. subterranea*. We did not attempt DNA extract analysis of TNS-F-185301 and TNS-F-185302 because the specimens lacked sufficient material to guarantee a DNA, and in our experience, such DNA is damaged due to repeated fumigation.

Genetic variations and geological distribution

The sequence of the D1-D2 region of 28S rDNA was almost identical to the available sequences from European specimens. The present sequence differed from EF608073,

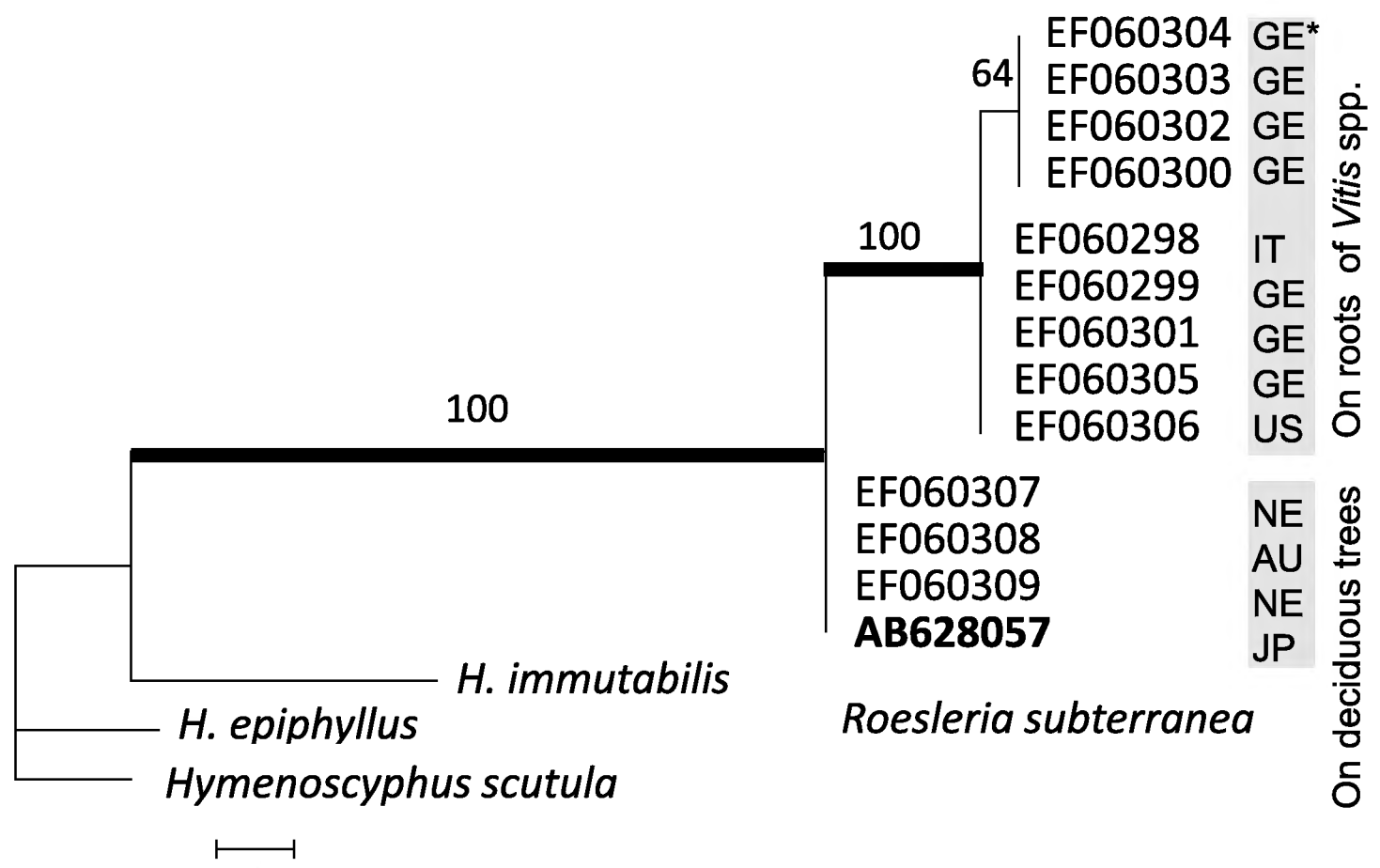


Figure 2. A single most parsimonious tree of *Roesleria subterranea* (ITS-5.8S sequences). Tree length = 31, CI = 0.8065, HI = 0.1935, RI = 0.8723, RC = 0.7035. The tree was constructed from a dataset of all available ITS-5.8S sequences of *R. subterranea* from various hosts and localities. The numbers on the branches indicate the bootstrap values (BP) of 1000 replications in maximum parsimony analysis when the BP exceeds 50%. The scale bar indicates the number of substitutions. Abbreviations indicate the localities of the isolates: AU, Austria; GE, German; IT, Italy; NE, Netherlands; US, USA; JP, Japan.

EF608074, and EF608075 at three, one, and four sites, respectively, among the 554 sites that were compared. The ITS-5.8S sequence was almost identical to the available sequences, and the identification based on morphology was confirmed.

In the MP analysis, we incorporated the available ITS-5.8S sequences of *R. subterranea*, using the three species of *Hymenoscyphus* used in Kirchmair et al. (2008) as outgroups. The aligned dataset included 466 characters, of which 28 characters were excluded from the analyses due to ambiguous alignment. The remaining 438 characters were used for the analysis, including a total of 23 parsimony-informative characters.

A single most parsimonious tree was obtained (Fig. 2). Within the tree, a monophyly of isolates from *Vitis* spp. from Germany, Italy and USA was strongly supported. The remaining group of this clade were found to be paraphyletic and composed of the isolates from various deciduous trees. The sequence obtained from the Japanese isolate (NBRC 108276) was included in this group (Fig. 2). Although the sequence of the latter group seemed to be identical in Fig. 2, the original sequence obtained from the Japanese isolate (AB628057) was not identical with several nucleotide differences to the other three. However, these differences were present only in the ambiguously aligned region, which was excluded from the analysis.

Although the tree topology depends on the choice of outgroup (data not shown), the monophyly of the species as a whole and the monophyly of the clade of isolates from *Vitis* (“*Vitis*-clade”) were highly stable (BS values were 100 in the present data and also in Kirchmair et al. (2008)). However, a group of isolates from other hosts besides *Vitis* (“non-*Vitis* group”) was paraphyletic. This result suggests that the host specificity was more important than the geographical distance as a factor for infraspecific genetic diversification.

All of the known isolates of the “*Vitis* clade” were obtained from cultivated grape samples. On the other hand, isolates of “non-*Vitis* grade” including our isolate were from the roots of *Malus sylvestris*, *Populus* sp. and other unknown wild deciduous trees. It is therefore hypothesized that isolates highly specific to *Vitis* are more widely distributed in the cultivated grape fields than is currently recognized. To clarify whether such a host specificity is present or not, inoculation experiments are indispensable. In addition, further isolates from various hosts and localities should be collected and examined to know the infraspecific relationships of the species.

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